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An anti-sulfatide antibody O4 immunoprecipitates sulfatide rafts including Fyn, Lyn and the G protein α subunit in rat primary immature oligodendrocytes

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Abstract The association of sulfatide with specific proteins in oligodendrocytes was examined by co-immunoprecipitation with an anti-sulfatide antibody. Protein kinase activity was detected in precipitates with a monoclonal antibody to sulfatide (O4) from the rat primary immature oligodendrocytes. We conducted *in vitro* kinase assay of tyrosine phosphorylated proteins of 80, 59, 56, 53 and 40 kDa by gel electrophoresis. Of these proteins, the proteins of 59 kDa and 53/56 kDa were identified as the Src family tyrosine kinases Fyn and Lyn on the basis of their sequential immunoprecipitation with anti-Fyn and anti-Lyn antibodies, respectively. The 40 kDa protein was identified as the α subunit of the heterotrimeric G protein. These observations suggest that O4 immunoprecipitates sulfatide rafts including Fyn, Lyn and the α subunit of the heterotrimeric G protein.

Keywords Sulfatide · O4 · Immunoprecipitation · Src family kinase · Oligodendrocytes

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Introduction

Galactosylceramide and sulfatide are major galactosphingolipid components of the oligodendrocyte plasma membrane and myelin [1–3]. Both galactosylceramide-null mice and sulfatide-null mice exhibit an aberrant enhancement of oligodendrocyte terminal differentiation and myelin dysfunction, suggesting a role for these galactosphingolipids in oligodendrocyte differentiation [4–7]. O4, an anti-sulfatide monoclonal antibody, was identified as a marker of late oligodendrocyte progenitors [8]. Furthermore, exposure of oligodendrocyte progenitors to O4 leads to the reversible arrest of oligodendrocyte lineage progression [9, 10]. The mechanism of O4-mediated regulation of oligodendrocyte differentiation remains to be fully understood.

We have been investigating the functional association of glycosphingolipids with signal transducers in the central nervous system [11–16]. We previously demonstrated that an anti-ganglioside GD3 antibody, R24, immunoprecipitates the Src family kinase Lyn from rat cerebellar granule cells. In this study, we investigated whether the anti-sulfatide antibody O4 immunoprecipitates signaling molecules such as Src family kinases from rat primary immature oligodendrocytes.

Materials and methods

Preparation of rat primary immature oligodendrocytes and immunocytochemistry

Primary immature oligodendrocytes were prepared as described previously [17]. In brief, the cerebral hemispheres from an 18-day-old rat embryo were enzymatically dissociated in a solution of dispase II (0.3 mg/ml, Boehringer Mannheim, Germany) and 0.05 % DNase (Boehringer Mannheim, Germany) in Dulbecco's

modified Eagle's medium (DMEM, GIBCO-BRL, MD). The dissociated cells were seeded on poly-L-lysine-coated culture dishes. After 7 days of culture, the cells were passaged with 0.25 % trypsin to remove nonoligodendrocyte lineage cells, and seeded in DMEM containing 10 % FCS to induce cell proliferation. After 7 days of culture, the cells were passaged with 0.25 % trypsin, and seeded in serum-free DMEM supplemented with 2 ng/ml bFGF to induce differentiation. After 7 days of culture, the cells were passaged with 0.25 % trypsin to remove mature oligodendrocytes, and seeded in serum-free DMEM. These procedures were repeated 3 times. A homogenous population of immature oligodendrocytes was isolated after 35 days of culture. For immunocytochemistry, cells were stained as previously described [18].

Immunoprecipitation and *in vitro* kinase assay

Immunoprecipitation with an anti-sulfatide monoclonal antibody (O4), and an *in vitro* kinase assay were performed as described previously [11]. In brief, 120,000 primary immature oligodendrocytes were solubilized in lysis buffer (0.5 % Triton X-100, 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Na3VO4, 1 mM EGTA, 1 mM phenylmethylsulfonylfluoride, 5 μg/mL leupeptin, and 5 μg/mL pepstatin A) at 4 °C for 20 min. Aliquots (0.5 ml) of the supernatants were incubated with O4 (2.5 μg/ml; R&D Systems MAB1326) or normal mouse IgM for 1 h, and then anti-mouse IgM goat antibody (2.5 μg/ml; Bethyl Laboratories A90-101A) for 1 h, and precipitated with protein G-Sepharose. Following immunoprecipitation, the *in vitro* kinase reaction was started by the addition of 5 μCi of [γ-^{32}P] ATP (3,000 Ci/mmol; NEN Life Science Products). Phosphorylation was stopped by the addition of Laemmli sample buffer, and the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by autoradiography. In a re-immunoprecipitation experiment, following the kinase reaction, the samples were boiled for 5 min in lysis buffer with 1 % SDS, diluted 10-fold with the lysis buffer, and then re-immunoprecipitated with antibodies to phosphotyrosine (PY20), Fyn (Fyn301), Lyn (Lyn8) and the α subunit of the heterotrimeric G protein internal (40–54) antibodies (Calbiochem).

Results and discussion

Detection of protein tyrosine kinase activity in immunoprecipitates with anti-sulfatide antibody (O4)

A large-scale homogeneous population of immature oligodendrocytes was isolated from an embryonic rat brain [17]. The immunocytochemical study showed that these cells were O4-immunoreactive and myelin basic protein (MBP)-negative (Fig. 1a, b). The immature oligodendrocytes differentiated to premyelinating oligodendrocytes, O4-immunoreative and MBP-immunoreative (Fig. 1d, e) by the addition of a conditioned medium of astrocytes [17]. Immunoprecipitates with the anti-sulfatide antibody (O4) from the Triton X-100 extract of the rat primary immature oligodendrocytes were analyzed for the presence of protein kinase activity by an *in vitro* kinase assay. *In vitro* kinase reaction resulted in the phosphorylation of several proteins of 80, 59, 56, 53 and 40 kDa, as determined by SDS-PAGE (Fig. 2, lane 2). No kinase activity was detected in immunoprecipitates with control mouse IgM (Fig. 2, lane 1). This phosphorylation was characterized by sequential immunoprecipitation with O4 and the anti-phosphotyrosine antibody. The *in vitro* kinase assay was performed using O4 immunoprecipitates, after which the immune complexes were disrupted by boiling in SDS-containing buffer and subjected to a second immunoprecipitation with the anti-phosphotyrosine antibody PY20. The anti-phosphotyrosine antibody precipitated the 80, 59, 56, 53 and 40 kDa proteins in re-immunoprecipitation experiments (Fig. 2, lane 3), suggesting that these proteins (p80, p59, p56, p53 and p40) are tyrosine-phosphorylated proteins.

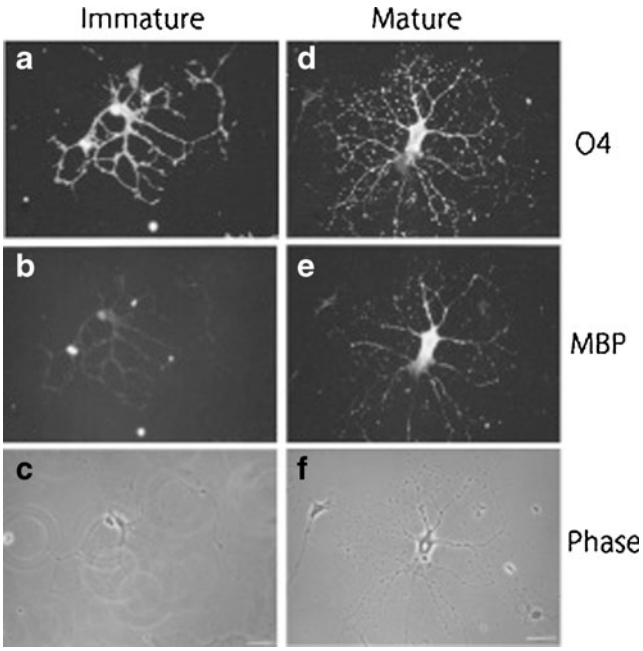

Fig. 1 Immunofluorescence analysis of cultured rat primary oligodendrocytes. Double-immunofluorescence staining of immature (**a**, **b**, **c**) and mature oligodendrocytes (**d**, **e**, **f**) with O4 (**a**, **d**) and anti-myelin basic protein antibodies (**b**, **e**) and phase-contrast cell morphology (**c**, **f**). *Bars* represent 22 μm

Identification of p59 and p53/56 as the Src family kinases Fyn and Lyn, respectively

The molecular weight and tyrosine phosphorylation of p59 and p53/56 suggested that they could be Src family tyrosine kinases. To investigate this possibility, we tried to identify them by sequential immunoprecipitation with O4 and anti-Src

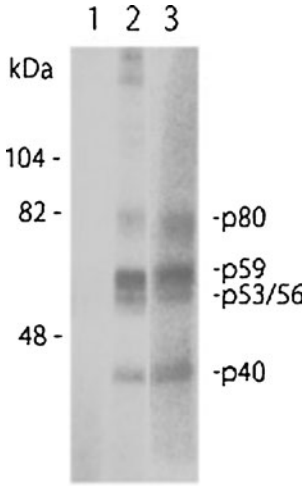

Fig. 2 Anti-sulfatide antibody O4-precipitated protein tyrosine kinase activity from rat primary immature oligodendrocytes. The cells were solubilized in lysis buffer. Supernatants were immunoprecipitated with the anti-sulfatide monoclonal antibody O4. Immunoprecipitates were subjected to *in vitro* kinase assay, SDS-PAGE. Phosphorylation was visualized by autoradiography. Precipitate with control mouse IgM (*lane 1*); precipitate with O4 (*lane 2*); eluted by boiling in 1 % SDS. After 10-fold dilution with lysis buffer, re-immunoprecipitation was carried out using the anti-phosphotyrosine antibody PY20 (*lane 3*)

family kinase antibodies. The anti-Fyn antibody and anti-Lyn antibody specifically precipitated p59 and p53/56 in re-immunoprecipitation experiments, respectively (Fig. 3).

The Src family kinase Fyn is involved in the oligodendrocyte differentiation process, because transgenic mice lacking Fyn exhibit a reduced number of oligodendrocytes and hypomyelination [19–22], and cultured oligodendrocytes from Fyn-deficient mice or those expressing dominant-negative Fyn show defects in the numbers of newly formed oligodendrocytes, as well as in the formation of complex branches of the myelin membrane [23, 24]. The substrate and binding-protein of Fyn also regulate oligodendrocyte differentiation [25, 26]. Fyn may be involved in the O4-mediated arrest of oligodendrocyte lineage progression. Furthermore, the GPI-anchored adhesion molecule F3/contactin transduces intracellular signals *via* Fyn in proteolipid protein-rich galactosphingolipid microdomains, membrane rafts, during myelination [27, 28]. Lyn contributes to proliferation signaling of oligodendrocyte progenitors [29]. Therefore, our findings suggest that O4 immunoprecipitates sulfatide rafts containing Fyn and Lyn, which are involved in oligodendrocyte differentiation.

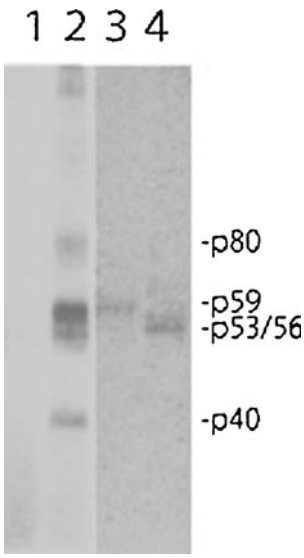

Fig. 3 Identification of p59 and p53/56 as Src family tyrosine kinases Fyn and Lyn. Precipitate with control mouse IgM (*lane 1*); precipitate with O4 (*lane 2*); eluted by boiling in 1 % SDS. After 10-fold dilution with lysis buffer, re-immunoprecipitation was carried out using the anti-Fyn antibody (*lane 3*) and anti-Lyn antibody (*lane 4*)

Identification of p40 as the α subunit of the heterotrimeric G protein

Previously, we demonstrated that the anti-ganglioside GD3 antibody R24 immunoprecipitates a 40 kDa protein, which we identified as the α subunit of the heterotrimeric G protein Go from rat cerebellar granule cells [14]. Therefore, we tried to identify p40 by sequential immunoprecipitation with O4 and the anti-α subunit of the heterotrimeric G protein antibody. p40 was re-immunoprecipitated with the antibody to the α subunit of the heterotrimeric G protein (Fig. 4). The oligodendrocyte-specific G protein-coupled receptor GPR17 orchestrates the transition between immature and myelinating oligodendrocytes [30]. Furthermore, a previous report showed the possible involvement of Gα13 in the proliferation of oligodendrocyte progenitors [31]. Therefore, heterotrimeric G protein signaling in membrane rafts may be involved in oligodendrocyte development. We also demonstrated that the α subunit of the heterotrimeric G protein may be tyrosine-phosphorylated in immature oligodendrocytes. Interestingly, a previous report showed the activation of the G protein through tyrosine phosphorylation of the α subunit by Fyn [32]. Furthermore, Src family kinases are a direct effector of

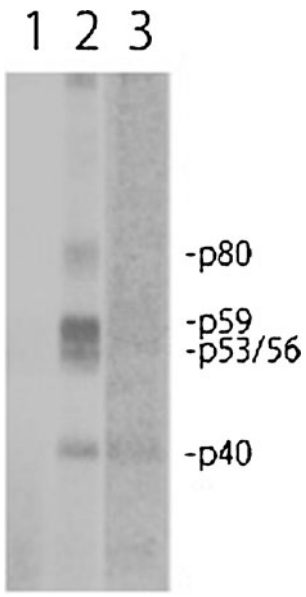

Fig. 4 Identification of p40 as trimeric G protein α subunit. Precipitate with control mouse IgM (*lane 1*); precipitate with O4 (*lane 2*); eluted by boiling in 1 % SDS. After 10-fold dilution with lysis buffer, re-immunoprecipitation was carried out using anti-G protein α subunit antibody (*lane 3*)

G proteins [33, 34]. These observations suggest the possible crosstalk of signaling molecules within membrane rafts. R24 co-immunoprecipitates not only Lyn and Gαo but also the GPI-anchored neural cell adhesion molecule TAG-1 and raft integral membrane protein Cbp [12, 16, 35]. TAG-1 is transiently expressed on premigratory cerebellar granule cells in the external granule cell layer. Ligation of TAG-1 induced Lyn activation and tyrosine phosphorylation of Cbp in primary cerebellar granule cells [12, 16]. On the other hand, treatment with SDF-1α, a ligand for the G protein-coupled receptor, stimulated GTPγS binding to Go and caused Goα translocation to the rafts, leading to the growth cone collapse of cerebellar granule cells [14]. The migration of cerebellar granule cells is impaired in TAG-1 or SDF-1α-deficient mice [36, 37]. These observations suggest that R24 can immunoisolate GD3 rafts including signal transducers, which are involved in the development of cerebellar granule cells.

Periventricular leukomalacia (PVL) is the principal form of brain injury in premature infants [38]. Lethal injury to premyelinating oligodendrocytes (preOLs; late oligodendrocytes progenitors, expressing chondroitin sulfate proteoglycan NG2 and O4, generated from oligodendrocyte precursor cells during embryonic development) in the immature cerebral white matter has been postulated to be a key feature of PVL, resulting in hypomyelination. Recent studies have also provided a new insight that O4 immunostaining indicates loss of premyelinating oligodendrocyte cell processes and abnormal O4-positive preOL-cell process morphology in PVL [39, 40]. The myelin abnormality in PVL might be due to a functional defect of sulfatide-rich raft signaling. Therefore, O4 may co-immunoisolate not only Fyn but also novel raft-signaling molecules involved in the differentiation of immature oligodendrocytes and myelin abnormality in PVL [3, 41, 42].

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Conflict of interest The authors declare no conflicts of interest.

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